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# CD11b and CD200 on Circulating Monocytes Differentiate Two Angiographic Subtypes of Polypoidal Choroidal Vasculopathy

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**PURPOSE.** To investigate surface expression of CD11b and CD200 on circulating monocytes in patients with polypoidal choroidal vasculopathy (PCV).

**METHODS.** This was a prospective case-control study of patients with PCV ( $n = 27$ ), age-matched healthy controls ( $n = 27$ ), and patients with neovascular AMD ( $n = 49$ ). All participants underwent a comprehensive ocular examination. Fluorescein and indocyanine green angiography were performed in patients suspected of neovascular AMD or PCV. Polypoidal choroidal vasculopathy was angiographically categorized into those with a strong presence of a branching vascular network (BVN) (type 1) or with a faint/no clear presence of a BVN (type 2). Fresh venous blood was stained with fluorescent antibodies for flow cytometric analyses. We compared the percentages of CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes between groups of diagnosis and between different angiographic subtypes of PCV.

**RESULTS.** Overall, CD11b<sup>+</sup> monocytes were both increased in patients with PCV and neovascular AMD. CD200<sup>+</sup> and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes were increased in patients with neovascular AMD. An age-related increase in CD11b<sup>+</sup>CD200<sup>+</sup> monocytes was absent in patients with PCV and neovascular AMD. Patients with PCV type 1 had significantly higher CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes, whereas patients with PCV type 2 had levels similar to that in healthy controls.

**CONCLUSIONS.** We found that PCV is immunologically heterogeneous with significant differences between angiographic subtypes. Increased CD11b<sup>+</sup> and CD200<sup>+</sup> monocytes in those with a strong presence of BVN indicate that BVN development may be associated with retinal injury and a VEGF-mediated process that is either reflected or propelled by systemic changes in monocytes.

**Keywords:** polypoidal choroidal vasculopathy, immunology, microglia, blood, monocytes

Age-related macular degeneration is the most common cause of irreversible vision loss in the elderly of the developed world.<sup>1</sup> The late stage of the disease includes an exudative phenotype where proliferating choroidal vessels break through the Bruch's membrane resulting in hemorrhage and exudate in the macula, which impairs visual acuity and function.<sup>2–4</sup> Although the etiology of neovascular AMD is not fully understood, growing evidence suggest a key role for chronic neuroinflammation and microglial activity (resident macrophages of the retina) that result in a proinflammatory environment wherein VEGF secretion is a drive-force for the choroidal neovascularizations (CNV).<sup>5–7</sup> An important differential diagnosis to neovascular AMD is polypoidal choroidal vasculopathy (PCV). Polypoidal choroidal vasculopathy was initially described by Yannuzzi<sup>8</sup> in 1982 as a variant of CNV in the peripapillary area, and since as a clinical entity distinctly different from neovascular AMD in choroidal originated polyp-

like structures with or without associated branching vascular network (BVN) typically under the RPE.<sup>9–12</sup> Although the clinical phenotype of PCV is clearly distinct from that of neovascular AMD, it is still debated whether they share similar etiology or also differ etiologically.<sup>12,13</sup> Tong et al.<sup>14</sup> found that intraocular levels of VEGF may be increased in PCV, but at a lower level than in neovascular AMD. Genetic studies suggest similarities and differences in the etiology of PCV and neovascular AMD.<sup>15–17</sup> A recent example is a missense variant in the FGD6 (FYVE, RhoGEF, and PF domain-containing protein 6) gene that contributes to angiogenesis only in PCV and not in neovascular AMD.<sup>17</sup> However, the amount of studies focusing on PCV is limited and etiologic aspects as well as similarities and differences between neovascular AMD and PCV remain to be understood.

Intravitreal VEGF inhibitors is a part of the treatment of PCV and VEGF is suspected to play an important role in the



etiology.<sup>12,14</sup> Interestingly, recent reports of treatment outcomes suggest that PCVs with a strong presence of BVN respond better to anti-VEGF treatment than PCVs without or very faint BVNs.<sup>18,19</sup> These results suggest that there may be differences in the etiologic mechanisms between these angiographic subtypes.<sup>20–23</sup> Indeed, genetic studies have found differences in the genetic background when comparing patients with these different angiographic subtypes, and patients with PCV with a strong presence of BVN are more likely to have a genetic composition similar to that seen in patients with neovascular AMD.<sup>20–22</sup> Polyps without or very faint BVN on the other hand have been associated with single nucleotide polymorphisms in the elastin gene suggesting a different etiologic process than that described for new vessel formations from the choroid.<sup>23</sup>

There is a growing interest in the role of circulating monocytes in AMD.<sup>24–28</sup> Macrophages (i.e., monocytes at site of injury that are differentiated into active phagocytes) possess both the capability of facilitating clearance of drusen and fostering an environment that eventually leads to CNV.<sup>25,29</sup> Their multifaceted function gives rise to conflicting hypotheses wherein they are the culprits, the saviors, or both.<sup>24,25</sup> Surface expression of CD11b and CD200 on circulating monocytes have previously been associated with neovascular AMD.<sup>24</sup> CD11b regulates the monocyte adhesion and migration processes in response to inflammation and CD11b<sup>+</sup> monocytes secrete VEGF at sites of inflammation significantly contributing to the formation of CNVs.<sup>29</sup> Activity of retinal microglia is regulated through CD200 receptor (CD200R) interaction with CD200 on neurons and monocytes.<sup>30,31</sup> CD200 inhibition leads to uncontrolled microglia activity and CD200 overexpression is a regulatory mechanism, whereby neurons and resident or infiltrating immune cells attenuate microglial activity.<sup>24,30–32</sup>

These etiologic aspects of circulating monocytes remain completely unexplored for PCV. Hence, in this study, we investigated the surface expression of CD11b and CD200 on circulating monocytes in patients with PCV and compared with healthy controls and patients with neovascular AMD.

## METHODS

This was a prospective case-control study of patients with PCV, patients with neovascular AMD, and healthy control individuals. We explained the nature of the study to all participants and obtained oral and written consent prior to participation. This study was approved by the Regional Committee of Ethics in Research of the Region of Zealand (SJ-379) and followed the principles stated in the Declaration of Helsinki.

### Study Participants

We recruited visitors to the Department of Ophthalmology at Zealand University Hospital Roskilde between February 2015 and February 2017 for this prospective case-control study. Patients with neovascular AMD or PCV were recruited consecutively from our outpatient program. Age-matched control individuals above 60 years of age were recruited among healthy visitors, who were primarily a biologically unrelated relative (e.g., husband or wife) to a recruited patient with neovascular AMD or PCV. This was an intentional strategy to match the control group in terms of lifestyles and exposures. Because no previous studies have investigated CD11b and CD200 in patients with PCV, power calculations were based on previous immunologic studies on patients with neovascular AMD.<sup>24</sup> Assuming an alpha level of 0.05, and a power level of 80%, we calculated that a sample size of 26 in each group was necessary to obtain sufficient power to detect

differences in CD11b<sup>+</sup>CD200<sup>+</sup> between the three groups of diagnosis.

### Retinal Diagnosis, Clinical Data, and Interviews

Participants were examined using slit-lamp biomicroscopy, digital fundus photography, and spectral-domain optical coherence tomography (SD-OCT). Best-corrected visual acuity (BCVA) was measured in each eye using the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart.<sup>33</sup> Healthy controls were defined as having less than 10 small drusen (diameter <63  $\mu$ m) without pigment abnormalities. Fluorescein and indocyanine green angiography (ICGA) was used for subtyping the disease to neovascular AMD or PCV. Neovascular AMD included cases with fibrovascular pigment epithelium detachments and CNV membranes with subretinal or sub-RPE hemorrhages or fibrosis. Polypoidal choroidal vasculopathy was diagnosed in cases with one or more polyps seen in the early-phase ICGA with a hypofluorescent halo and with or without BVN. The ICGA video was used to evaluate the pulsation of polyps. Other PCV characteristics were not mandatory for the diagnosis, but were used to support the final diagnosis (fundus examination revealing orange-red focal subretinal polyp-like structures and retinal OCT showing a protrusion from the choroid elevating RPE from the Bruch's membrane). We further categorized PCVs into subtypes based on ICGA presence of BVN: type 1, polypoidal lesions with visible feeder and draining vessels and strong presence of BVNs; or type 2, polypoidal lesions without clear visible feeder or draining vessels and without or faint BVNs.<sup>18,19,21–23</sup>

All participants were interviewed to obtain a list of medical conditions, and ongoing treatments and medications. Data were crosschecked with the patient's electronic patient record. Smoking habits was categorized in current smokers, previous smokers (smoked >100 cigarettes during lifetime and ceased smoking >12 months), or never smokers. Alcohol use was reported in units/week (1 unit = 12-mL ethanol). Physical activity was assessed using a single question on regular activity.<sup>34,35</sup> Height and weight was measured to calculate body mass index.

### Participant Eligibility

Eligible participants had either healthy retinas, neovascular AMD in one or both eyes, or PCV in one or both eyes. Because we measured the systemic immune phenotype of the individuals, we restricted our study population to individuals without ongoing immune diseases that can affect our results and blur our conclusions. Thus, we did not include participants with a diagnosis of cancer, autoimmune disease, or any immune, infectious, or inflammatory diseases, or patients who were receiving chemotherapy or any immune modulating therapy. We also did not include participants that had received VEGF inhibitors (anti-VEGF) within the last 4 or 8 weeks for ranibizumab and aflibercept, respectively, to avoid potential interaction of systemic antibodies in the following preparations. Retinal diagnosis including retinal angiography was made on treatment-naïve eyes, but patients were not recruited on their first visit as onset of CNV is associated with acute immune activity.<sup>36</sup> Eligible patients were identified and recruited from follow-up visits. We post-hoc excluded participants with elevated plasma C-reactive protein levels as this is a sign of ongoing immune disease.<sup>37</sup>

### Blood Sampling, Preparation, and Flow Cytometry

Venous blood from antecubital veins were sampled in an EDTA-coated tube for flow cytometric analyses and a lithium heparin

coated tube for serum CRP measurement. Flow cytometry was performed within 4 hours after blood sampling. We used an automated hematology analyzer Sysmex KX-21N (Sysmex Corporation, Kobe, Japan) to obtain the white blood cell count, which was used to calculate the volume of blood needed to obtain  $5 \times 10^5$  white blood cells in each test tube. The red blood cells were lysed using 1% red blood cell lysis buffer (Nordic Biosite AB, Täby, Sweden) for 10 minutes at room temperature and in the dark. Cells were washed in an isotonic buffer (BD FACSFlow; BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged for 5 minutes at 500g. This process was repeated three times, after which the cells were resuspended in the isotonic buffer. We then added the following monoclonal antibodies: Allophycocyanine-CY7 CD11b, IgG1  $\kappa$ , Clone ICRF44, Cat. No. 557754 (BD Biosciences); Phycoerythrin-CY7 CD14, IgG2a  $\kappa$ , Clone M5E2, Cat. No. 301814 (BioLegend, San Diego, CA, USA); and Phycoerythrin CD200, IgG1  $\kappa$ , Clone 325516, Cat. No. FAB27241P (R&D Systems, Inc., Minneapolis, MN, USA). Fluorochrome-matched negative isotypes were added to a separately prepared tube: Allophycocyanine-CY7 IgG1  $\kappa$ , Clone MOPC-21, Cat. No. 400128 (BioLegend); Phycoerythrin-CY7 IgG2a  $\kappa$ , Clone MOPC-173, Cat. No. 400232 (BioLegend); and Phycoerythrin IgG1  $\kappa$ , Clone MOPC-21, Cat. No. 555749 (BD Biosciences). Incubation was done at room temperature in the dark for 20 minutes, after which the cells were washed and resuspended in the isotonic FACSFlow buffer. Stained cells were analyzed using flow cytometry (BD FACSCanto II; BD Biosciences) with a sample size gated for 100,000 singlet leukocytes. Flow data were analyzed using Kaluza Analysis (Kaluza Analysis v. 1.5.20365.16139; Beckman Coulter, Inc., Pasadena, CA, USA) by two independent evaluators (YS and MKN) who were blinded to the conditions of the participants. We used cell height and area to gate singlets and size and granularity forward scatter [FSC]/side scatter [SSC] to gate monocytes, which was then used to investigate expression of the markers investigated (Fig. 1). Nonspecific signaling was eliminated using corresponding negative isotype controls with a threshold of 1%.

### Data Analysis and Statistics

All statistics were performed using SPSS 23 (IBM Corporation, Armonk, NY, USA) and presented using Prism 7 (GraphPad, La Jolla, CA, USA). Normally distributed continuous data were presented in mean and SD and compared using parametric tests; otherwise data were presented in median and interquartile range (IQR) and compared using nonparametric tests. Categorical variables were presented in numbers and percentages and tested using  $\chi^2$  test, and we used the Fisher's exact test when dealing with small categories. We compared the frequencies of monocytes that were CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> between the groups. To evaluate the size of the observed differences between the groups, we calculated Cohen's *d*, which is defined as the ratio between the group difference and the SD for the data and interpreted following the suggestions by Cohen and Sawilowsky: <0.2 (negligible/very small), 0.2 (small), 0.5 (moderate), 0.8 (large), and 1.2 (very large).<sup>38,39</sup> We explored immunosenescence (i.e., age-dependent changes of the immune system) for CD11b<sup>+</sup>CD200<sup>+</sup> monocytes, for which we previously found an age-related increment in healthy individuals that was absent in patients with neovascular AMD.<sup>24</sup> Finally, we explored differences in CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes among patients with PCV stratified by their angiographic subtype. *P* values below 0.05 were interpreted as sign of statistical significance.

### RESULTS

We recruited 31 healthy controls, 27 patients with PCV, and 54 patients with neovascular AMD for this study during the enrollment period. All participants were Caucasians. Due to high plasma C-reactive protein levels (>15 mg/L), indicating a possible ongoing acute immune response, four healthy controls and five patients with neovascular AMD were excluded. Thus, our analyses include a total of 103 participants consisting of 27 healthy controls, 27 patients with PCV, and 49 patients with neovascular AMD. Time from initial retinal diagnosis for patients with PCV or neovascular AMD to study recruitment and blood sampling were median 16 (IQR: 4–36) months and median 18 (9–38) months, respectively, for patients with PCV and patients with neovascular AMD. Groups did not differ in demographics, co-morbidities, or lifestyle factors (Table 1), but differed significantly in CD11b<sup>+</sup> monocytes ( $P < 0.001$ , one-way ANOVA), CD200<sup>+</sup> monocytes ( $P = 0.009$ , one-way ANOVA), and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes ( $P = 0.014$ , one-way ANOVA).

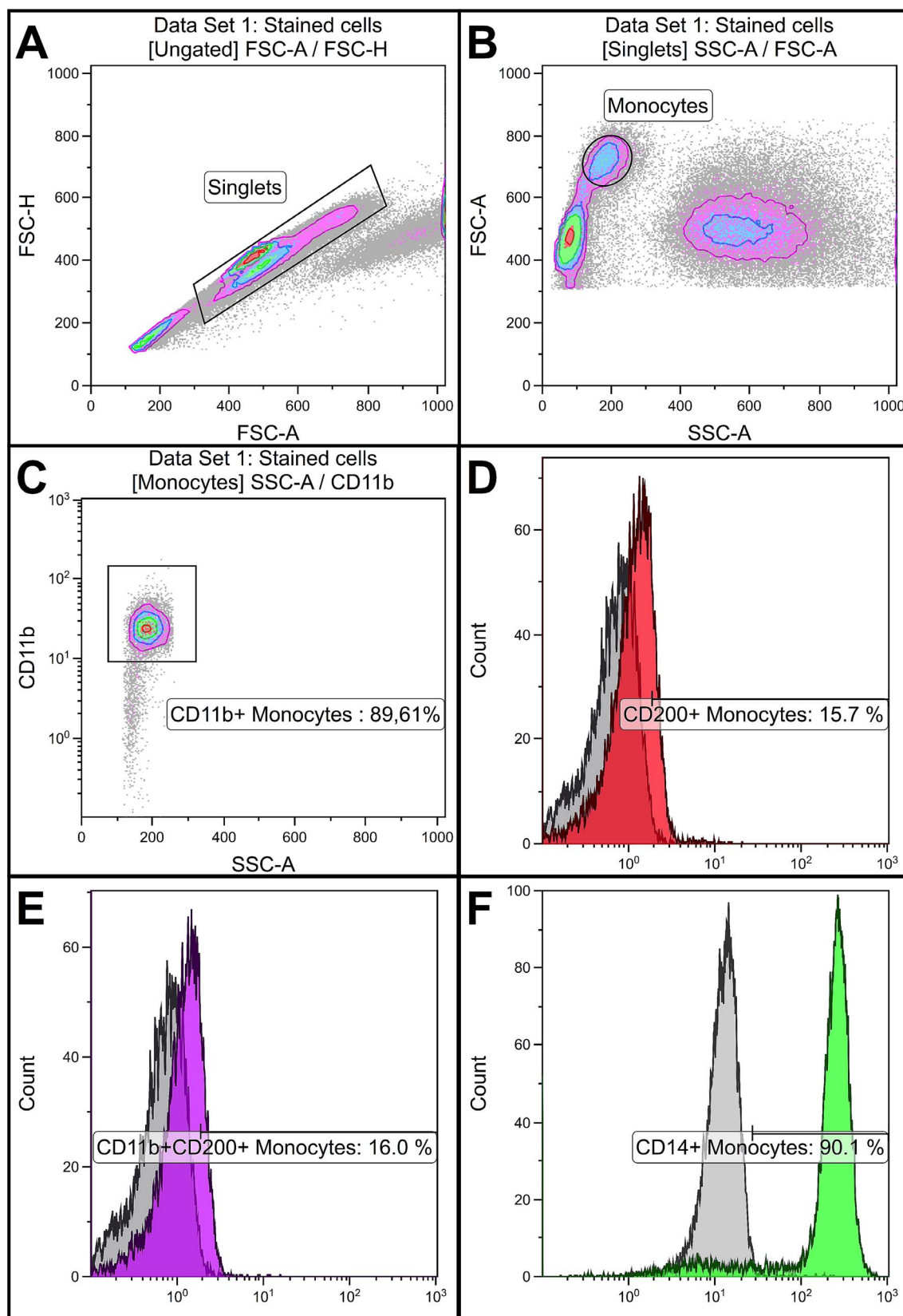
Patients with PCV had significantly higher CD11b<sup>+</sup> monocytes than healthy controls, but CD200<sup>+</sup> monocytes did not differ (Fig. 2). Percentage of CD11b<sup>+</sup> monocytes was significantly higher than in healthy controls (mean difference: 2.9%, confidence interval [CI] 95%: 0.8%–5.0%,  $P = 0.008$ , independent samples *t*-test). Percentage of CD200<sup>+</sup> monocytes did not differ significantly from healthy controls (mean difference: 0.8%, CI 95%: –4.0% to 5.7%,  $P = 0.740$ , independent samples *t*-test). Percentage of CD11b<sup>+</sup>CD200<sup>+</sup> monocytes also did not differ significantly from healthy controls (mean difference: 0.%, CI 95%: –4.2% to 5.9%,  $P = 0.735$ , independent samples *t*-test). Time from retinal diagnosis to blood sampling (duration of disease) did not correlate with percentages of CD11b<sup>+</sup> monocytes ( $\rho = 0.10$ ,  $P = 0.604$ , Spearman's correlation), CD200<sup>+</sup> monocytes ( $\rho = 0.04$ ,  $P = 0.838$ , Spearman's correlation), or CD11b<sup>+</sup>CD200<sup>+</sup> monocytes ( $\rho = 0.05$ ,  $P = 0.796$ , Spearman's correlation).

Patients with neovascular AMD had significantly higher CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes compared with healthy controls (Fig. 2). Percentage of CD11b<sup>+</sup> monocytes was significantly higher than in healthy controls (mean difference: 5.2%, CI 95%: 3.1%–7.2%,  $P < 0.001$ , independent samples *t*-test). Percentage of CD200<sup>+</sup> monocytes was significantly higher than in healthy controls (mean difference: 6.3%, CI 95%: 2.0%–10.7%,  $P = 0.005$ , independent samples *t*-test). Percentage of CD11b<sup>+</sup>CD200<sup>+</sup> monocytes was also significantly higher than in healthy controls (mean difference: 6.4%, CI 95%: 1.7%–11.0%,  $P = 0.008$ , independent samples *t*-test). Time from retinal diagnosis to blood sampling (duration of disease) did not correlate with percentages of CD11b<sup>+</sup> monocytes ( $\rho = -0.10$ ,  $P = 0.478$ , Spearman's correlation), CD200<sup>+</sup> monocytes ( $\rho = 0.08$ ,  $P = 0.601$ , Spearman's correlation), or CD11b<sup>+</sup>CD200<sup>+</sup> monocytes ( $\rho = 0.08$ ,  $P = 0.578$ , Spearman's correlation).

Patients with PCV differed significantly from patients with neovascular AMD in CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> (Fig. 2). Patients with neovascular AMD had a significantly higher percentage of CD11b<sup>+</sup> monocytes (mean difference: 2.3%, CI 95%: 0.9%–3.6%,  $P = 0.001$ , independent samples *t*-test), CD200<sup>+</sup> monocytes (mean difference: 5.5%, CI 95%: 0.3%–10.7%,  $P = 0.037$ , independent samples *t*-test), and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes (mean difference: 5.5%, CI 95%: 0.1%–11.0%,  $P = 0.048$ , independent samples *t*-test).

Effect sizes of the group differences are shown in Table 2. The difference in CD11b<sup>+</sup> monocytes between patients with PCV and healthy controls had a moderate size (Cohen's *d*: 0.7). The difference in CD11b<sup>+</sup> monocytes between patients with neovascular AMD and healthy controls had a very large size





**FIGURE 1.** Gating strategies to identify CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes using flow cytometric data in Kaluza software. First, we identified singlets (**A**) and then monocytes using forward versus side scatter (**B**). Then, CD11b<sup>+</sup> monocytes were identified (**C**). The CD200<sup>+</sup> monocytes were less bright, and the percentage of CD200<sup>+</sup> monocytes were determined using antibody corresponding negative isotype controls at 1% to distinguish positive cell populations from nonspecific background signals (**D**). The CD11b<sup>+</sup>CD200<sup>+</sup> co-expressing monocytes were measured by identifying CD200<sup>+</sup> monocytes among CD11b<sup>+</sup> monocytes (**E**). We looked at CD14<sup>+</sup> expression on the monocyte population to confirm that the population investigated was indeed monocytes (**F**).

TABLE 1. Participant Characteristics

	Patients With PCV ( <i>n</i> = 27)	Healthy Controls ( <i>n</i> = 27)	Patients With nAMD ( <i>n</i> = 49)	<i>P</i> Value
<b>Demographics</b>				
Age, y mean (SD)	72.3 (7.6)	74.3 (7.5)	75.7 (7.4)	0.156
Sex, <i>n</i> , females:males	16:11	17:10	24:25	0.448
<b>Co-morbidities</b>				
Hypertension, <i>n</i> (%)	11 (41)	10 (37)	25 (51)	0.448
Cardiovascular disease, <i>n</i> (%)	5 (19)	3 (11)	11 (22)	0.494
Hypercholesterolemia, <i>n</i> (%)	9 (33)	9 (33)	12 (25)	0.615
Type 2 diabetes, <i>n</i> (%)	3 (11)	0 (0)	6 (12)	0.201
<b>Lifestyle factors</b>				
Smoking, <i>n</i> (%)				0.288
Current	8 (30)	4 (15)	15 (31)	
Previous	15 (55)	13 (48)	21 (43)	
Never	4 (15)	10 (37)	13 (26)	
Alcohol consumption, units, median (IQR)	4 (1-13)	4 (2-7)	3 (1-9)	0.703
Body mass index, mean (SD)	25.4 (4.2)	25.5 (3.4)	26.3 (3.9)	0.537
Physically active, <i>n</i> (%)	15 (56)	17 (63)	25 (51)	0.605

(Cohen's *d*: 1.3), whereas for the CD200<sup>+</sup> and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes the sizes were moderate (Cohen's *d*: 0.6 and 0.6, respectively). The differences in CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes between patients with PCV and patients with neovascular AMD were of moderate size (Cohen's *d*: 0.6, 0.5, and 0.5, respectively).

In healthy controls, we found a positive correlation between age and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes ( $\rho = 0.41$ ,  $P = 0.035$ , Spearman's correlation) (Fig. 3). This age-related upregulation of CD11b<sup>+</sup>CD200<sup>+</sup> was absent in patients with PCV ( $\rho = -0.04$ ,  $P = 0.854$ , Spearman's correlation) and in patients with neovascular AMD ( $\rho = 0.03$ ,  $P = 0.852$ , Spearman's correlation) (Fig. 3), which indicated that CD11b<sup>+</sup>CD200<sup>+</sup> monocytes in patients with PCV may not be completely similar to that seen in healthy controls as indicated by comparisons between groups of diagnosis.

Patients with PCV type 1 (*n* = 15) differed significantly from those with type 2 (*n* = 12): CD11b<sup>+</sup> monocytes were significantly higher (mean difference: 2.85%, CI 95%: 0.9%-

4.8%,  $P = 0.008$ , independent samples *t*-test), CD200<sup>+</sup> monocytes were significantly higher (mean difference 11.1%, CI 95%: 4.5%-17.6%,  $P = 0.002$ , independent samples *t*-test), and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes were significantly higher (mean difference 11.3%, CI 95%: 4.5%-18.0%,  $P = 0.002$ , independent samples *t*-test). Interestingly, CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> measurements in patients with PCV type 1 were similar to those in patients with neovascular AMD, whereas measurements in patients with PCV type 2 were similar to those in healthy controls (Fig. 4). Patients with PCV type 1 did not differ from those with type 2 in terms of demographics, co-morbidities, or lifestyle factors ( $P > 0.05$  for all comparisons).

# DISCUSSION

Angiogenesis and CNV formation depend on monocyte dynamics.<sup>27,28</sup> In this study, we find evidence of altered phenotype of circulating monocytes in patients with PCV

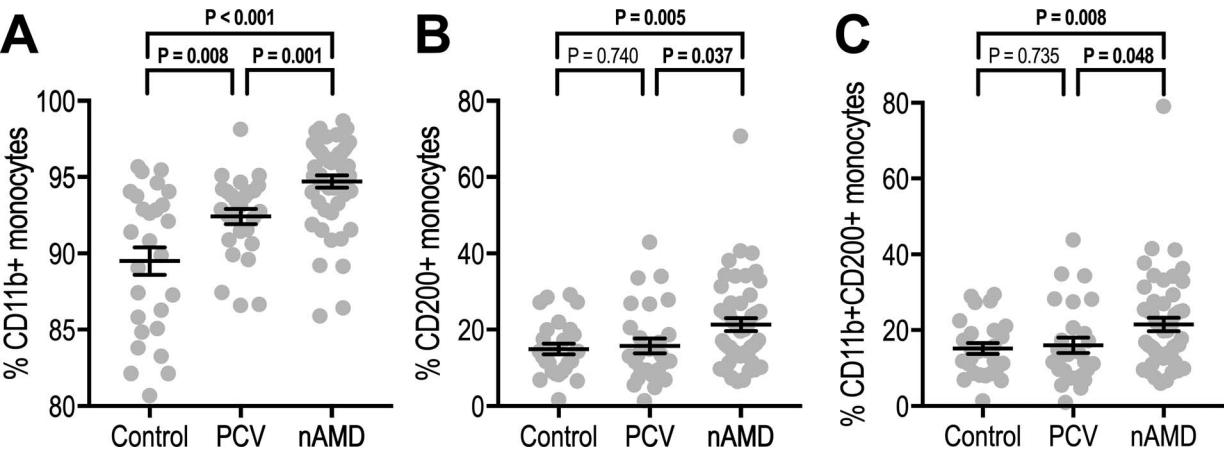


FIGURE 2. CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes in healthy controls (control), patients with PCV, and patients with neovascular AMD (nAMD). Whiskers represent mean and  $\pm$  standard error. (A) Patients with PCV had more CD11b<sup>+</sup> monocytes than controls. Patients with nAMD had more CD11b<sup>+</sup> monocytes than both controls and patients with PCV. (B) Patients with PCV did not differ significantly in CD200<sup>+</sup> monocytes compared with controls. Patients with nAMD had significantly more CD200<sup>+</sup> monocytes than controls and patients with PCV. (C) Patients with PCV did not differ significantly in CD11b<sup>+</sup>CD200<sup>+</sup> monocytes compared with controls. Patients with nAMD had significantly more CD11b<sup>+</sup>CD200<sup>+</sup> monocytes than controls and patients with PCV.

TABLE 2. Size of Differences (Cohen's *d*) Between the Groups

	CD11b <sup>+</sup> Monocytes	CD200 <sup>+</sup> Monocytes	CD11b <sup>+</sup> CD200 <sup>+</sup> Monocytes
Patients with PCV vs. healthy controls	0.7 (moderate)	0.1 (very small)	0.1 (very small)
Patients with PCV vs. Patients with nAMD	0.6 (moderate)	0.5 (moderate)	0.5 (moderate)
Patients with nAMD vs. healthy controls	1.3 (very large)	0.6 (moderate)	0.6 (moderate)

Cohen's *d* is calculated as the ratio between the difference between the groups and the SD for the data. Its interpretation is suggested by Cohen and Sawilowsky to the following: <0.2 (negligible/very small), 0.2 (small), 0.5 (moderate), 0.8 (large), and 1.2 (very large).

depending on angiographic subtype. Strong presence of BVN was associated with a higher percentage of CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes, at a level similar to that seen in patients with neovascular AMD, which suggests that VEGF and microglial activity play a role in BVN development. In contrast, no such changes were found in patients with PCV without or a very faint BVN, which from an immunologic perspective were similar to our healthy controls. These findings suggest that PCV constitute a heterogeneous disease entity, clinically as well as immunologically, wherein some aspects are similar to neovascular AMD while others differ.

Crosstalk between systemic circulating immune cells and the microglia is important for maintaining neuronal health.<sup>25,40</sup> In the aged, anatomic, and physiological changes of the retina lead to an environment characterized by an increased need for phagocytosis of cellular by-products and lipoproteins in the outer retina.<sup>41,42</sup> Increased neuronal stress and injury in the outer retina forces retinal microglia to migrate from inner to outer retina.<sup>43,44</sup> As the resident phagocytes of the retina, the microglia increases their activity but may fail to keep up with the increasing phagocytic demand and retinal stress with age.<sup>43,44</sup> This leads to an environment where circulating monocytes are increasingly recruited and macrophages accumulate in the retina to keep up with the need for phagocytosis and increased retinal stress.<sup>26,45</sup> However, this balance in the retina wherein the monocytes comes in as the saviors is overthrown in the event of CNV formation in the late stage of AMD. Experimental models of laser-induced CNV demonstrate that monocytes infiltrate and determine the aftermath of the injury.<sup>27,28</sup> Interestingly, experimental depletion of monocytes attenuates the size of CNV lesions,<sup>27,28</sup> and similar positive correlation between monocytes and lesion size are seen in patients with recent onset of neovascular AMD.<sup>36</sup> Contributing to a view of

monocytes as the culprits of the story, specific monocytic phenotypes in the systemic circulation have been associated with neovascular AMD.<sup>24,46,47</sup> Elucidating characteristics of these monocytic phenotypes is important to understand how immunologic ageing and dysfunction contributes to the etiology of AMD.

Ageing leads to an increased systemic expression of IL-10, which through Signal transducer and activator of transcription (STAT3)-mediated pathway polarizes macrophages into the proangiogenic M2 or CD14<sup>high</sup>CD16<sup>+</sup> intermediate phenotypes.<sup>46–48</sup> Blocking IL-10R or STAT3 leads to attenuated CNV in experimental models confirming the significance of these age-related monocyte/macrophage changes to the development of CNVs.<sup>46,47</sup> These proangiogenic CD14<sup>high</sup>CD16<sup>+</sup> monocytes have a higher expression of CD11b and adhere up to seven times more efficiently to vascular cell adhesion molecule 1,<sup>49,50</sup> which is upregulated locally in eyes with neovascular AMD.<sup>51</sup> These CD11b<sup>+</sup> macrophages are a significant source of VEGF.<sup>29</sup> Interestingly, presence of complement component 3 regulates CD11b expression and increased level of complement component 3 fragment C3a is found in patients with neovascular AMD.<sup>52,53</sup> Taken together, these studies suggest that neovascular AMD is linked to a combination of immunologic ageing and dysfunction, and accordingly it is not surprising that our patients with neovascular AMD have a substantial increase in systemic levels of CD11b<sup>+</sup> monocytes compared with age-matched healthy controls. In patients with PCV, CD11b<sup>+</sup> monocytes were only moderately increased; however, delving into clinical details revealed that the level of CD11b<sup>+</sup> monocytes did not differ significantly from the high levels seen in patients with neovascular AMD when focusing on patients with PCV type 1, whereas the level of CD11b<sup>+</sup> monocytes in patients with PCV type 2 was comparable to healthy controls. Considering

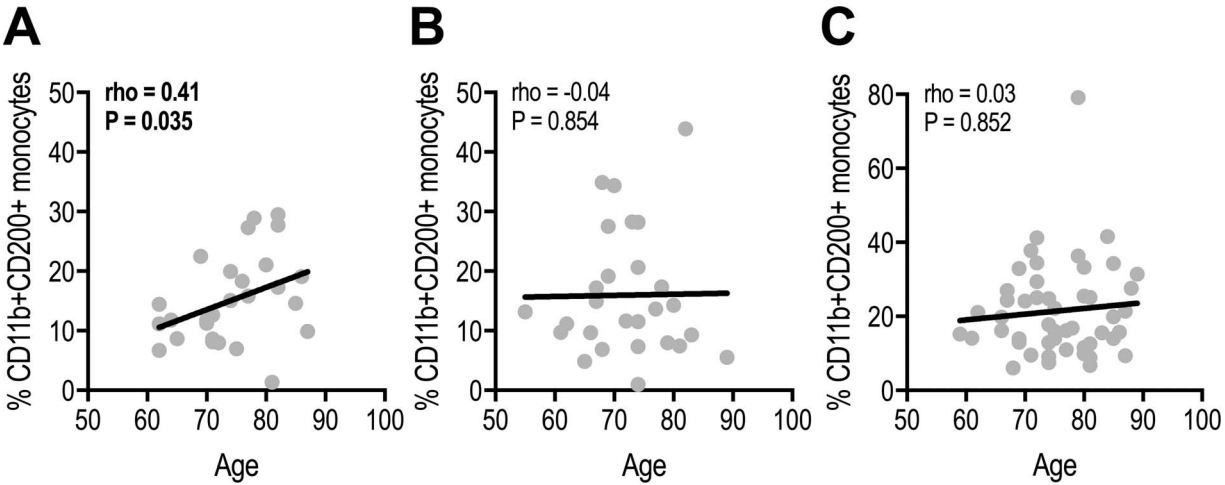
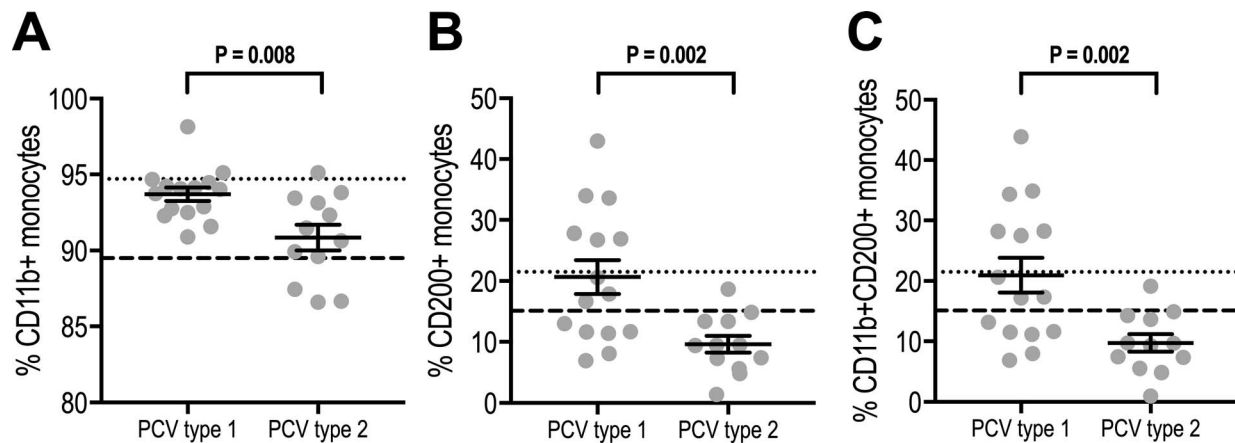


FIGURE 3. Scatterplots to explore relationship between CD11b<sup>+</sup>CD200<sup>+</sup> (y-axis) and increasing age (x-axis) in healthy controls (A), patients with PCV (B), and patients with neovascular AMD (C) using Spearman's correlation. In patients with PCV and in patients with neovascular AMD, no significant relationships were observed. In healthy controls (B), there was a positive correlation between age and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes.



**FIGURE 4.** CD11b<sup>+</sup>, CD200<sup>+</sup>, and CD11b<sup>+</sup>CD200<sup>+</sup> monocytes in patients with PCV stratified by angiographic subtype at time of diagnosis. *Whiskers* represent mean and  $\pm$  standard error. Corresponding mean of healthy controls (*dasbed line*) and mean of patients with nAMD (*dotted line*) are included for reference. Polypoidal choroidal vasculopathy type 1 was associated with higher CD11b<sup>+</sup> (A), CD200<sup>+</sup> (B), and CD11b<sup>+</sup>CD200<sup>+</sup> (C) monocytes when compared with PCV type 2.

that CD11b<sup>+</sup> macrophages are a strong source of VEGF and that BVNs consists of newly formed choroidal vessels, results of this study suggest that VEGF may play a more substantial role for the disease development in PCV type 1.

Signaling between neurons and monocytes/macrophages through CD200:CD200R interaction can modulate microglial and macrophage activity.<sup>30–32</sup> Experimental autoimmune retinitis leads to increased CD200R expression on microglia and increased CD200 expression on neurons and infiltrating cells, which suggests that CD200:CD200R interaction is important in orchestrating a regulated microglial and macrophage response.<sup>31</sup> It is shown in CD200 knockout mice that microglia becomes highly present and active in the retina,<sup>54</sup> which confirms the regulatory role of CD200:CD200R interaction on the microglia. In this current study, we find that CD200<sup>+</sup> monocytes and CD200<sup>+</sup> on CD11b<sup>+</sup> monocytes are increased in patients with neovascular AMD, which is in line with a previous study.<sup>24</sup> Considering the regulatory function of CD200, upregulation of systemic CD200 in patients with neovascular AMD may be a regulatory mechanism to dampen an increased microglial activity due to retinal stress, inflammation, and CNV. Our study adds to this knowledge as patients with PCV were found to differ in their CD200<sup>+</sup> monocytes when stratified by angiographic subtype, suggesting strong presence of BVN may be associated with an increased microglial activity.

We previously found that ageing was correlated with CD200<sup>+</sup> on CD11b<sup>+</sup> monocytes in healthy individuals,<sup>24</sup> possibly due to increased microglial activity with age that is counteracted by systemic regulation. This correlation was missing in patients with neovascular AMD likely due to generally upregulated CD200<sup>+</sup> on monocytes. In this study, we also found a correlation between age and CD11<sup>+</sup>CD200<sup>+</sup> monocytes in healthy controls. Such correlations were lacking in patients with PCV as well as in patients with neovascular AMD, possibly reflecting an upregulation due to an increased microglial activity.

Vascular dysfunction may play a significant role in PCV. Jones et al.<sup>55</sup> investigated previous associations between HTRA1 and PCV by transgenically expressing human HTRA1 in mice RPE by which the mice developed choroidal-originated BVN and polypoidal lesions. Zeng et al.<sup>56</sup> found that matrix metalloproteinases 2 and 9, which regulate turnover of extracellular tissue, were increased in the serum of patients with PCV compared with early AMD, neovascular AMD, and

healthy controls. These studies indicate that there is a significant component of vascular dysfunction in PCV. Histopathologic examinations by Nakashizuka et al.<sup>57</sup> identified hyalinization of the choroidal vessels with foamy macrophages similar to that seen atherosclerosis. Interestingly, binding of LDL to CD14<sup>high</sup>CD16<sup>+</sup> intermediate proangiogenic circulating monocytes is a key component in the differentiation toward foamy macrophages.<sup>58</sup>

Taken together, we propose that our results and conclusions from previous studies suggest an overall picture of PCV type 1 as a vascular originated disease propelled by systemic changes, which manifests as vascular pathologies primarily at sub-RPE level; whereas neovascular AMD can be considered as an intraretinal originated disease propelled by systemic changes, which manifests as vascular and retinal pathologies at intra- and subretinal level. In contrast, PCV type 2 may be a vascular originated disease with a limited amount of retinal damage. Future studies need to confirm and elaborate on these hypotheses.

It should be noted that our observational study design, wherein we find associations and correlations does not necessarily imply causation, which requires experimental verification. We remain to elucidate whether the observed changes in the circulatory system reflect a systemic adaptation to retinal injury and microglial dysfunction, or whether the changes reflect a fundamental disease in the immune system that in the event of retinal stress causes vascular growth. Another limitation is that our study uses a Caucasian population and its generalizability to non-Caucasian populations may be complicated by PCV being subject to considerable interethnic differences as demonstrated in its epidemiology, clinical characteristics, and genetic associations.<sup>12,13,59,60</sup>

In conclusion, we find that PCV from an immunologic perspective constitute a heterogeneous disease entity with important systemic differences between those with and without BVN. Changes in CD11b and CD200 on monocytes may reflect that BVN in PCV is a VEGF-mediated process that is associated with an increased microglial activity or response, whereas polyps without or very faint BVNs may differ substantially in their underlying etiology. These findings shed light on the mechanisms of a poorly understood disease and suggest that there may not be a simple answer to the question of whether or not PCV differs etiologically from AMD.



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